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RESEARCH ARTICLE

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Molecular Markers for Detecting a Wide Range of *Trichoderma* spp. that Might Potentially Cause Green Mold in *Pleurotus eryngii*

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ABSTRACT

In *Pleurotus* sp., green mold, which is considered a major epidemic, is caused by several *Trichoderma* species. To develop a rapid molecular marker specific for *Trichoderma* spp. that potentially cause green mold, eleven *Trichoderma* species were collected from mushroom farms and the Korean Agricultural Culture Collection (KACC). A dominant fungal isolate from a green mold-infected substrate was identified as *Trichoderma pleuroticola* based on the sequences of its internal transcribed spacer (ITS) and translation elongation factor 1-α (*tef1*) genes. In artificial inoculation tests, all *Trichoderma* spp., including *T. atroviride*, *T.* cf. *virens*, *T. citrinoviride*, *T. harzianum*, *T. koningii*, *T. longibrachiatum*, *T. pleurotum*, and *T. pleuroticola*, showed pathogenicity to some extent, and the observed symptoms were soaked mycelia with a red-brown pigment and retarded mycelium regeneration. A molecular marker was developed for the rapid detection of wide range of *Trichoderma* spp. based on the DNA sequence alignment of the ITS1 and ITS2 regions of *Trichoderma* spp. The developed primer set detected only *Trichoderma* spp., and no cross reactivity with edible mushrooms was observed. The detection limits for the PCR assay of *T. harzianum* (KACC40558), *T. pleurotum* (KACC44537), and *T. pleuroticola* (CAF-TP3) were found to be 500, 50, and 5 fg, respectively, and the detection limit for the pathogen-to-host ratio was approximately 1:10,000 (wt/wt).

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1. Introduction

Pleurotus eryngii (king oyster mushroom) is widely consumed in East Asia and has increased in popularity in Europe and North America [1,2] due to its remarkable flavor and high nutritional value [3,4]. Thus, the significance and production of this mushroom are increasing, and the main countries that produce this mushroom are China (1,360,000 M/T, 2015) (Korea Agriculture Trade Information, KATI, http://www.kati.net), Korea (49,136 M/T, 2018, Ministry of Agriculture, Food and Rural Affairs, http://www.mafra.go.kr/), and Japan (39,411 M/T, 2018, Forestry Agency of Japan, http://www.rinya.maff.go.jp/).

P. eryngii is cultivated using a polypropylene (PP) bottle system involving filling PP bottles with media, sterilization with steam, cooling, inoculation, spawn running, scraping old spawn, fruiting, and harvest [5]. Many mushroom farms for *P. eryngii* production are well built with sanitation systems and automation [6], but diseases and pests are still serious threats to high quality and stable production.

A wide range of *Trichoderma* species have been found to be associated with green mold in *Pleurotus* spp. Specifically, *T. harzianum* and *T. aggressivum* exhibit potent virulence to *Pleurotus* spp., as demonstrated by considerable yield losses and quality reductions [7,8]. *T. atroviride*, *T.* cf. *virens*, *T. citrinoviride*, *T. koningii, T. longibrachiatum, T. pleurotum*, and *T. pleuroticola* have also been reported as causal agents of *Pleurotus* [8–11]. Additionally, *T. pleurotum* and *T. pleuroticola* have been found to suppress the growth of *P. eryngii* [12] *in vitro*.

The genus *Trichoderma* is ubiquitous in various environments, including soil, forest, and root ecosystems [13], and several *Trichoderma* species have even been isolated from a natural habitat and a fruiting body of *Pleurotus* [10]. However, controlling the diseases caused by *Trichoderma* could be challenging due to the difficulty in distinguishing pathogenic mycelia from mushroom mycelia at the early growth stage because both are white. Moreover, *Trichoderma* spp. produce large numbers of conidia during asexual development [13], which can be transferred throughout mushroom farms by ventilation and workers because these species are airborne

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and have a high ability to adhere to clothes [14]. Monitoring the *Trichoderma* population in incubation rooms and scraping rooms is thus critical. Therefore, the development of an effective detection method for *Trichoderma* spp. in both mycelial cultures and environments is important for decreasing yield losses during mushroom cultivation.

In this study, we collected *Trichoderma* spp. from infected substrates and microorganism stock centers and investigated their pathogenicity. A molecular marker was then developed for the rapid and specific detection of *Trichoderma* spp.

2. Materials and methods

2.1. Fungal strains and growth conditions

A total of 11 *Trichoderma* strains were used, one was isolated in this work, and the others were from microorganisms stock center. Fungal isolates from substrate of bottles infected with green mold in *P. eryngii* cultivation farm in Jinju, Korea, were isolated by diluting and plating the sampled material on mushroom complete media (MCM; 0.2% peptone, 0.2% yeast extract, 2.0% glucose, 0.05% $MgSO_4{\cdot}7H_2O$, 0.05% K_2HPO_4, and 0.046% KH_2PO_4). The plates were incubated at 25.0 °C until fungal mycelia appeared, and the mycelia were then transferred to fresh MCM medium for identification. Dominant and dark greenish fungi were selected for further experiments. Ten strains of *Trichoderma* spp. were provided by the Korean Agricultural Culture Collection (KACC), Jeonju, Korea (Table 1). *P. eryngii*, *P. ostreatus*, and *Grifola frondosa* were obtained from the Gyeongnam Agricultural Research and Extension Services, Jinju, Korea (Table 1), and used as controls in the pathogenicity test and PCR assays. All fungal isolates and strains were maintained on MCM in the dark at 25 °C with periodic transfers.

2.2. Genomic DNA extraction and sequencing of ITS and TEF-1α regions

The fungal genomic DNA (gDNA) was extracted from lyophilized mycelia using a GenEx Plant *plus!* Kit (GeneAll, Seoul, Korea) as described previously [15]. The internal transcribed spacer 1 (ITS1), 5.8S rRNA, and ITS2 regions were amplified by PCR using the ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) primers [16]. The *tef1* gene was amplified using the translation elongation factor 728 (TEF728) (GCCATCCTTGGAGATACCAGC) and TEF1 (CATCGAGAAGTTCGAGAAGG) primers [17] in a 20-µl reaction mixture as described previously [18]. The PCR products were analyzed in a 1.0% agarose gel (Promega, Madison, WI, USA) containing SafeView Classic (Intron Biotechnology, Seongnam, Korea). The specific bands were then cut out and sequenced [19]. For each reaction, at least three different amplified genes were bidirectionally sequenced. The sequence that corresponds to *tef1* gene was identified by an alignment with identical genes from GenBank. The sequences obtained in this study have been deposited in GenBank under the accession number MK611085 (ITS) and MT219315 (*tef1*). The ITS and *tef1* sequences were identified using *Trich*OKEY 2 [20] and *Tricho*BLAST [21] in combination with the NCBI-BLAST database.

2.3. Pathogenicity test

Trichoderma spp. were grown on MCM at 25 °C for 14 days, and the conidia were harvested with distilled water. The suspensions were filtered through a nylon membrane (40 µm; Millipore, Billerica, MA, USA) and resuspended to about 1.4×10^8 conidia per ml, as determined by direct counting using a hemocytometer.

Table 1. List of *Trichoderma* spp. and mushrooms used in this study.

Accession and strain no.	Fungal species	Source of isolation	Location of isolation
KACC[a]40558	*Trichoderma harzianum*	*Pleurotus ostreatus*	Buyeo, Chungnam, Korea
KACC40563	*Trichoderma longibrachiatum*	*Pleurotus ostreatus*	Buyeo, Chungnam, Korea
KACC40774	*Trichoderma atroviride*	Oyster mushroom cultivation field	Paju, Gyeonggi, Korea
KACC40779	*Trichoderma koningii*	Mushroom cultivation field	Paju, Gyeonggi, Korea
KACC40783	*Trichoderma* cf. *virens*	–	Gimcheon, Gyeongbuk, Korea
KACC40784	*Trichoderma harzianum*	Cotton waste on oyster mushroom cultivation field	Yeoju, Gyeonggi, Korea
KACC44535	*Trichoderma pleuroticola*	Agaric medium, rice straw	Danyang, Chungbuk, Korea
KACC44536	*Trichoderma pleuroticola*	Agaric medium, sawdust	Yangju, Gyeonggi, Korea
KACC44537	*Trichoderma pleurotum*	Agaric medium, sawdust	Paju, Gyeonggi, Korea
KACC44703	*Trichoderma citrinoviride*	*Lentinula edodes*	Goheung, Jeonnam, Korea
CAF-TP3[b]	*Trichoderma pleuroticola*	*Pleurotus eryngii*	Jinju, Gyeongnam, Korea
KNR[c]2312	*Pleurotus eryngii*	N/A[d]	N/A
KNR2183	*Pleurotus ostreatus*	N/A	N/A
KNR9020	*Grifola frondosa*	N/A	N/A

[a]KACC: Korea Agricultural Culture Collection accession number.
[b]CAF-TP3: Korea National College of Agriculture and Fisheries Mushroom Stock.
[c]KNR: Gyeongnam Rural Development Administration strain number.
[d]N/A: Not Applicable.

The fructification of *P. eryngii* KNT2312 was analyzed as reported previously [22]. At the end of the mycelial run (35 days at 25 °C), the old spawn and top layer of substrate (~1 cm) were removed by scraping to induce fruiting. Subsequently, 1 ml of *Trichoderma* solution containing approximately 1.4×10^8 conidia was inoculated onto the substrate surface in each PP bottle, and the cultures were placed in a room that was maintained at 15 °C with 95% relative humidity and cool-white fluorescence light (200 Lux). The control culture was inoculated with distilled water. The fruiting bodies were harvested before the pileus had opened completely to determine various phenotypic traits, including the yield, pileus, and stipe, using calipers (Mitutoyo, Tokyo, Japan) and a balance as described previously [22]. The earliness (days to harvest after removing old medium) was also measured. Four repetitions were performed for each of the 11 *Trichoderma* spp. and the control.

The statistical significance of the yield and earliness in the presence of different *Trichoderma* spp. was determined by comparing the group means through analysis of variance (ANOVA) followed by Duncan's multiple-range test (DMRT). Statistically significant differences were determined at $p < 0.05$. The data analyses were performed using R open-source software [23].

2.4. Primer design for potential causative *Trichoderma spp*

The sequences of the ITS regions of *Trichoderma* spp. with those of edible mushrooms were compared using Clustal Omega software to identify specific sequences for *Trichoderma* spp. [24]. In addition to the ITS sequence of *T. pleuroticola* (CAF-TP3), additional ITS sequences were retrieved from GenBank: *T. pleuroticola*, EU918140; *T. pleuroti*, NR_134421; *T. pleurotum*, EU280069; *T. aggressivum*, AY154947; *T. aureoviride*, AF194007; *T. harzianum*, EU918151; *P. eryngii*, FJ90770; *P. ostreatus*, FN391585; *Agaricus bisporus*, AJ409229; *Flammulina velutipes*, KJ999151; and *Lentinula edodes*, AF079572. The specific sequences were used to design specific primers targeting the consensus sequences in the ITS1 and ITS2 regions of *Trichoderma* with the aim of amplifying only those of *Trichoderma* spp. and not those of edible mushrooms. The developed primer sequences were TDP-F: 5′-CGAGTTTACAACTCCCAAA-3′ and TDP-R: 5′-GAAAGTTGGGTGTTTAACG-3′. The primer set was tested by *in silico* PCR on ITS sequences of *Apergillus* and *Penicillium* from GenBank (NR_077154.1, NR_077145.1, NR_111348.1, NR_111041.1, NR_151784.1) for selectivity using FastPCR software [25].

2.5. PCR conditions, sensitivity, and specificity of PCR assays

PCR was performed using Ex Taq (Takara, Kyoto, Japan) and the following conditions: initial denaturation at 98 °C for 30 s, 35 cycles of denaturation at 98 °C for 10 s, annealing at 60 °C for 30 s, and extension at 72 °C for 1 min, and one cycle of final extension at 72 °C for 7 min. The PCR products were resolved on a 1% agarose gel containing SafeView Classic. To assess the diagnostic sensitivity, 10-fold serial dilutions of *T. harzianum* (KACC40558), *T. pleurotum* (KACC44537), and *T. pleuroticola* (CAF-TP3) gDNA, ranging from 5 ng to 5 ag per 20-μl PCR, were prepared. To evaluate the specificity of the PCR, serially diluted mixtures (10-fold) of host:pathogen gDNA ranging from 1:10 to 1:100,000 (wt/wt) were prepared: *P. eryngii* KNR2312 gDNA with *T. harzianum* (KACC40558), *T. pleurotum* (KACC44537) or *T. pleuroticola* (CAF-TP3) gDNA. The PCRs were performed under the same aforementioned conditions.

3. Results

3.1. Identification of greenish fungus from substrate infected with green mold

The dominant fungi appeared to grow rapidly on MCM and exhibited dark green mycelia. Isolates (CAF-TP3) were selected based on their morphology compared with the typical morphology of *Trichoderma* spp. The ITS1 and ITS2 regions were amplified by PCR using the universal primers ITS1 and ITS4. The sequence of the amplicon was 639 bp and shared 98.6% identity with other *T. pleuroticola* in NCBI. The *Trich*OKEY v.2.0 identification profile showed three barcode sequences in ITS1 and ITS2: GATCTCTG, GTTTTTTTATAATCT, and CCCCTCGTGGG. These barcode sequences identified the isolate as *T. pleuroticola* with high reliability in the *Trich*OKEY.

PCR using the TEF728 and TEF1 primer set amplified a single band of 2237 bp. The sequence corresponding to *tef1* gene was estimated to be 613 bp long. As determined with TrichoMARK, the sequence of the *tef1* gene of the CAF-TP3 contained two phylogenetic markers, namely, 301 bp of tef_int4 (large) and 86 bp of tef1_int5 (short), which share 99.3% and 100% with other *T. pleuroticola* in NCBI, respectively. In addition, 3 of 12 known *tef* anchors, EF1-986r (GGCAAGGGTT), tef1fw (GTGAGCGTG), and EF2 (ACTGGTAC), were found in the *tef1* sequence.

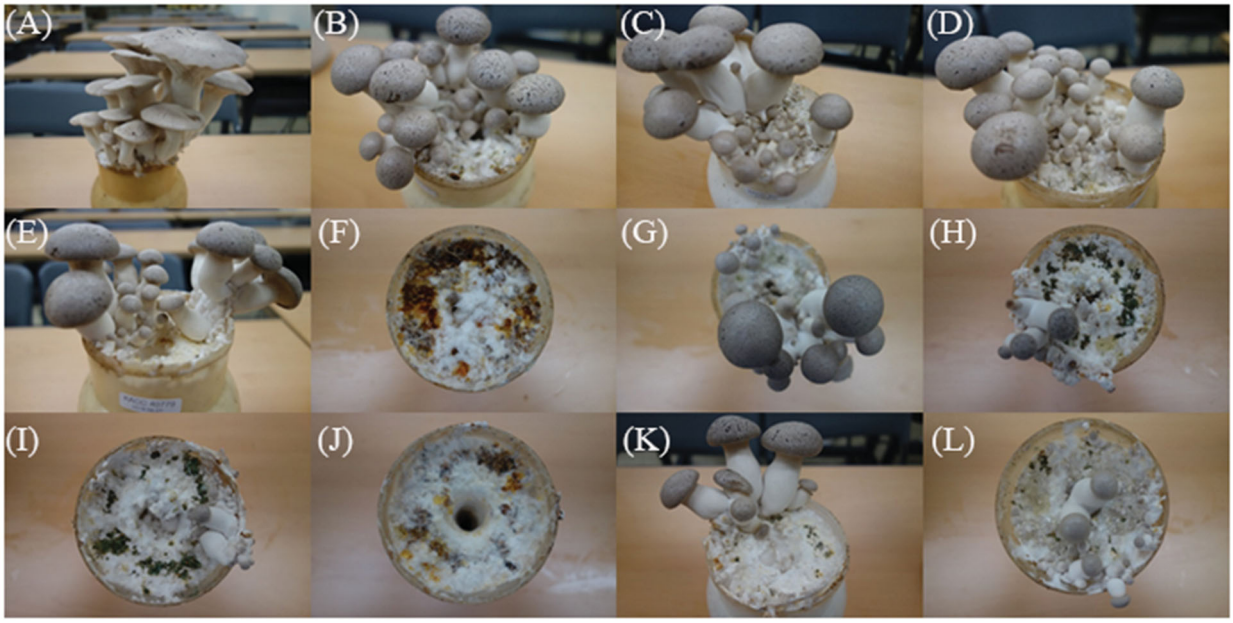

Figure 1. Symptoms of green mold in the fruiting body of *P. eryngii* after artificial inoculation with *Trichoderma* spp. (A) Control (distilled water); (B) *T. harzianum* (KACC40558); (C) *T. longibrachiatum* (KACC40563); (D) *T. atroviride* (KACC0774); (E) *T. koningii* (KACC40779); (F) *T.* cf. *virens* (KACC40783); (G) *T. harzianum* (KACC40784); (H) *T. pleuroticola* (KACC44535); (I) *T. pleuroticola* (KACC44536); (J) *T. pleurotum* (KACC44537); (K) *T. citrinoviride* (KACC44703); and (L) *T. pleuroticola* (CAF-TP3). Each inoculation was performed with 1.4×10^8 conidia. Picture were taken 18 days after inoculation.

3.2. Pathogenicity of Trichoderma spp. to P. eryngii

Trichoderma strains used in this study need to be ensure their pathogenicity to *P. eryngii* because they were isolated from various sources and their severity of green mold was obscure, even though all were associated with green mold. The appearance and development of the fruiting body and green mold symptoms were monitored daily. At the initial stage of fruiting body development, mycelia covered the substrate and formed condensed mycelia that became primordia. However, the *Trichoderma*-inoculated substrates were not covered or were slowly covered with mycelia. The grown mycelia of *P. eryngii* produced a reddish-brown pigment on all substrates after inoculation with *Trichoderma* spp. The main symptoms were soaked mycelia and retarded mycelial regeneration, which resulted in lack of fruiting, yield loss, and late harvest. Primordia did not appear on the substrate inoculated with *T. pleurotum* KACC44537, whereas on the substrate inoculated with *T.* cf. *virens* KACC40783, primordia appeared in a few areas (Figure 1(F,J)). Small fruiting bodies and late harvests were observed with the remaining *Trichoderma* species (Figure 1(A–E,G–I,K,L)). All *Trichoderma* species caused yield loss, ranging from 20.5% to 99.4% compared with that obtained with the control, and late harvest (Table 2). Prolonged earliness was observed on all the substrates treated with *Trichoderma* species, ranging from 0.3 to 6.0 days compared with that obtained with the control. *T. harzianum* (KACC40784) did not cause significant yield loss (238.6 g vs. 240.0 g) but led to late harvest (20.8 vs. 17.0 days) (Table 2). The statistical analysis showed that the yield and earliness were not significantly different between the treatments. However, the yields obtained in the three *Trichoderma*-treated plots (*T. pleurotum* KACC44537, *T. atroviride* KACC40774, and *T.* cf. *virens* KACC40783) were significantly lower than that of the control ($p < 0.05$), and the measured earliness in six *Trichoderma*-treated plots was statistically significantly later than that of the control. Moreover, the quality and hardness of the fruiting bodies were lower than those of the control. After *Trichoderma* infection, a reddish brown lesion was found inside the base stipe, and the shelf life of the fruiting body after infection was shorter compared with that of the control (data not shown).

3.3. Detection performance, sensitivity, and specificity of the developed primer set

A sequence alignment analysis showed the ITS regions of *Trichoderma* spp. and edible mushrooms (Figure 2). Several forward and reverse primer sets were designed based on the consensus sequences flanking the 18S rRNA and 28S rRNA sequences (Figure 2). The developed PCR sets were tested using *Trichoderma* spp. and several edible mushroom gDNA extracts. A single amplicon was observed for each *Trichoderma* sp. at the expected size (~530 bp) with the TDP-F and TDP-R primer combination, and no cross-reactivity could be

Table 2. Phenotypic characteristics of *Pleurotus eryngii* artificially infected with *Trichoderma* spp.

	Pileus		Stipe			
Inoculated *Trichoderma* spp.	Diameter (mm)	Length (mm)	Diameter (mm)	Length (mm)	Yield (g/bottle)*	Earliness (days)†
N/I	72.2 ± 14.4	31.7 ± 9.8	35.9 ± 6. 7	67.7 ± 12.6	240.0 ± 38.7^{a}	17.0 ± 0.0^{f}
T. harzianum KACC40558	54.2 ± 12.3	26.1 ± 10.6	38.6 ± 10.4	57.7 ± 13.9	181.4 ± 50.1^{ab}	17.8 ± 0.0^{def}
T. longibrachiatum KACC40563	67.3 ± 20.3	27.8 ± 8.8	35.0 ± 9.1	65.0 ± 10.7	197.6 ± 115.1^{ab}	17.5 ± 0.6^{def}
T. atroviride KACC40774	57.2 ± 11.6	21.4 ± 4.4	32.7 ± 6.4	62.7 ± 12.0	143.4 ± 62.6^{bc}	19.0 ± 1.8^{bcde}
T. koningii KACC40779	54.8 ± 17.6	23.4 ± 6.7	33.7 ± 8.2	62.2 ± 14.1	194.2 ± 58.7^{ab}	17.3 ± 0.5^{ef}
T. cf. *virens* KACC40783	36.9 ± 6.1	1.95 ± 3.7	34.2 ± 12.1	28.2 ± 11.5	49.2 ± 8.9^{c}	23.0 ± 0.0^{a}
T. harzianum KACC40784	51.7 ± 16.7	25.7 ± 6.5	32.3 ± 7.4	65.5 ± 24.2	238.5 ± 66.1^{ab}	20.8 ± 1.5^{b}
T. pleuroticola KACC44535	63.8 ± 15.0	38.4 ± 12.3	38.8 ± 7.2	83.3 ± 13.4	203.6 ± 78.4^{ab}	19.3 ± 1.5^{bcd}
T. pleuroticola KACC44536	76.2 ± 1.0	29.1 ± 6.3	41.8 ± 8.2	60.6 ± 22.2	169.2 ± 75.8^{ab}	20.0 ± 1.4^{bc}
T. pleurotum KACC44537	N/F	N/F	N/F	N/F	N/F	N/F
T. citrinoviride KACC44703	63.2 ± 18.4	25.6 ± 6.1	41.3 ± 12.6	64.6 ± 16.3	204.0 ± 28.1^{ab}	18.5 ± 1.1^{cdef}
T. pleuroticola CAF-TP3	78.2 ± 8.9	37.3 ± 11.3	50.2 ± 9.1	77.7 ± 9.3	181.2 ± 69.4^{ab}	18.7 ± 1.8^{cdef}

N/I: no inoculation; N/F: no fruiting body.
*Yield (g/bottle), yield per 1100-ml PP bottle.
†Earliness, days to harvest after removal of old medium. Means with the same letter in the yield and earliness columns were not significantly different at $p < 0.05$ according to Duncan's multiple range test (DMRT); the values are the means from four bottles.

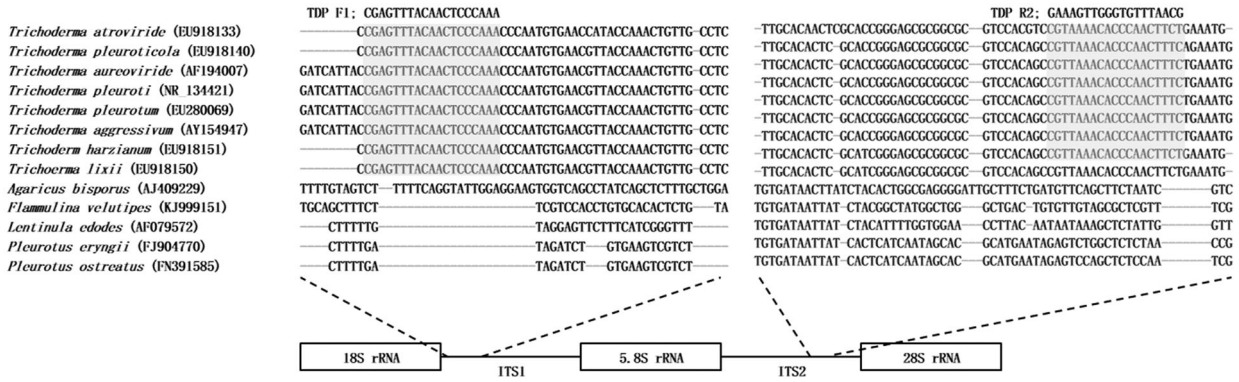

Figure 2. Sequence comparison of the ITS1 and ITS2 regions of *Trichoderma* spp. and edible mushrooms. The conserved regions of *Trichoderma* spp. are shaded in gray in the sequences corresponding to the specific primer set TDP-F and TDP-R.

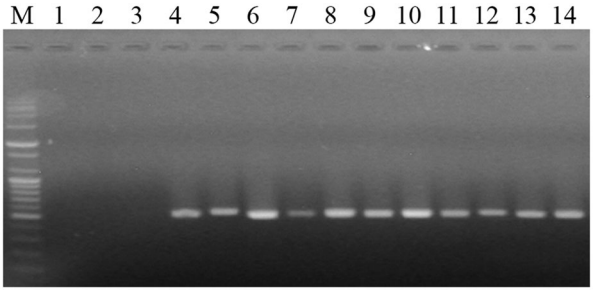

Figure 3. DNA fragments amplified by PCR using the TDP-F and TDP-R primer set specific for *Trichoderma* spp. M, 100-bp plus DNA ladder (Bioneer, Daejeon, Korea); lane 1, *Pleurotus eryngii*; lane 2, *P. ostreatus*; lane 3, *Grifola frondosa*; lane 4, *T. harzianum* (KACC40558); lane 5, *T. longibrachiatum* (KACC40563); lane 6, *T. atroviride* (KACC40774); lane 7, *T. koningii* (KACC40779); lane 8, *T.* cf. *virens* (KACC40783); lane 9, *T. harzianum* (KACC40784); lane 10, *T. pleuroticola* (KACC44535); lane 11, *T. pleuroticola* (KACC44536); lane 12, *T. pleurotum* (KACC44537); lane 13, *T. citrinoviride* (KACC44703); and lane 14, *T. pleuroticola* (CAF-TP3).

observed with the edible mushrooms (Figure 3). In addition, the primer set showed no amplicon with genome sequences of *Aspergillus* and *Penicillum* in *in silico* PCR (data not shown).

The application of the TDP-F and TDP-R primer set to the 10-fold dilution series of *T. harzianum* (KACC40558), *T. pleurotum* (KACC44537), and *T. pleuroticola* (CAF-TP3) gDNA consistently revealed detection limits of 50 fg, 500 ag, and 5 fg, respectively, after 35 cycles of amplification (Figure 4).

PCR using the developed primer set could amplify a single amplicon at a pathogen:*P. eryngii* ratio as low as 1:10,000 for all tested *Trichoderma* spp. (Figure 5). Weak amplicons were observed at a ratio of 1:100,000, but the band was likely unreliable.

4. Discussion

In this study, we isolated a dark green fungus from a substrate of *P. eryngii* infected with green mold and identified it as *T. pleuroticola*. Three barcode sequences from ITS sequences and two phylogenetic markers from the *tef1* gene, tef_int4 (large) and tef1_int5 (short), confirmed that the isolate was *T. pleuroticola*. *T. harzianum* and its biotypes are known as severe pathogens to *A. bisporus* [26], whereas *T. pleurotum* and *T. pleuroticola* are frequently found in *Pleurotus* mushroom farms [9–11] but not in *P. eryngii*. To our knowledge, this study describes the first isolation of *T. pleuroticola* from a substrate of *P. eryngii*.

All *Trichoderma* species showed pathogenicity, as demonstrated by a lack of fruiting bodies and

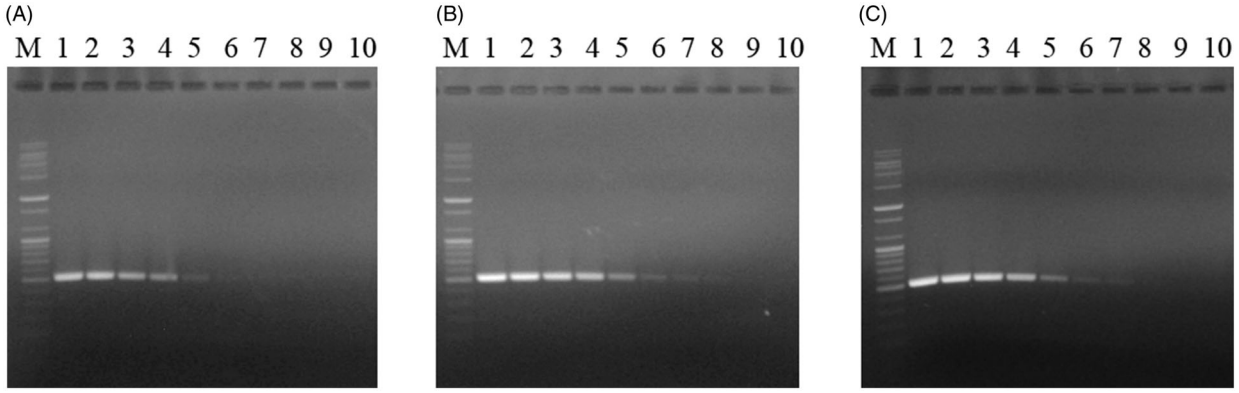

Figure 4. Sensitivity of the TDP-F and TDP-R primer set for pathogen DNA extracts. DNA bands amplified by PCR with the TDP-F and TDP-R primer set from 10-fold serial dilutions of gDNA from 5 ng to 5 ag of (A) *T. harzianum* (KACC40558); (B) *T. pleurotum* (KACC44537); or (C) *T. pleuroticola* (CAF-TP3). M, 100-bp plus DNA ladder (Bioneer, Daejeon, Korea); lane 1, 5 ng; lane 2, 500 pg; lane 3, 50 pg; lane 4; 5 pg; lane 5, 500 fg; lane 6, 50 fg; lane 7, 5 fg; lane 8, 500 ag; lane 9, 50 ag; and lane 10, 5 ag.

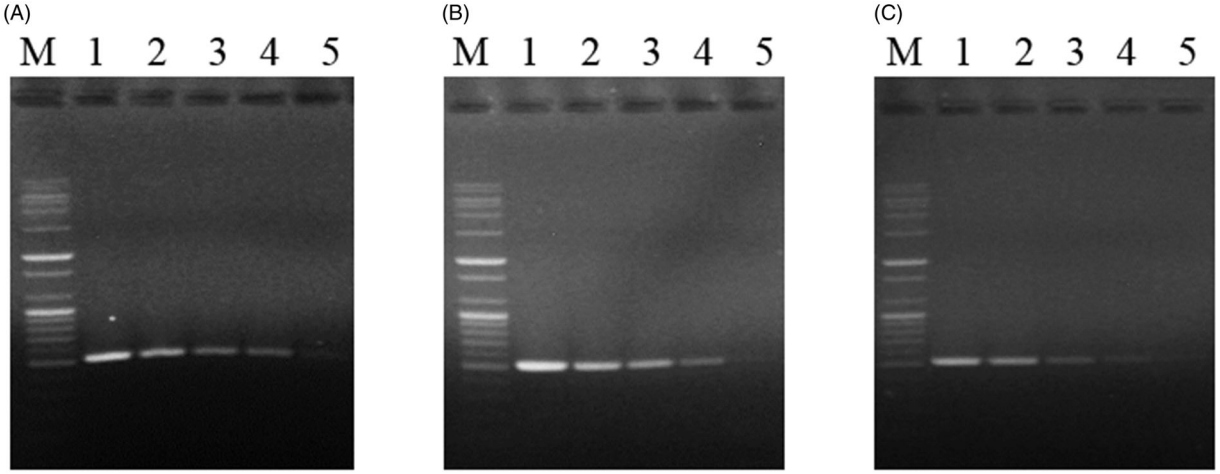

Figure 5. Specificity of the TDP-F and TDP-R primer set for host–pathogen DNA mixtures. DNA bands amplified by PCR with the TDP-F and TDP-R primer set from serially diluted mixtures (10-fold) of *P. eryngii* KNR2312 gDNA with gDNA from (A) *T. harzianum* (KACC40558); (B) *T. pleurotum* (KACC44537); or (C) *T. pleuroticola* (CAF-TP3) prepared at ratios ranging from 1:10 to 1:100,000 (wt/wt). M, 100-bp plus DNA ladder (Bioneer, Daejeon, Korea); lane 1, 1:10 (wt/wt) ratio of pathogen:*P. eryngii*; lane 2, 1:100 (wt/wt) ratio of pathogen:*P. eryngii*; lane 3, 1:1000 (wt/wt) ratio of pathogen:*P. eryngii*; lane 4; 1:10,000 (wt/wt) ratio of pathogen:*P. eryngii*; and lane 5, 1:100,000 (wt/wt) ratio of pathogen:*P. eryngii*.

soaked lesions. Interestingly, *T.* cf. *virens* and *T. pleurotum*, which are not known for their pathogenic severity, even though *T.* cf. *virens* was previously found to inhibit the mycelial growth of *P. eryngii* [27], caused no or very less fruiting body formation (Table 2). *T. pleurotum* and *T. pleuroticola* have also been reported to reduce the mycelial growth of *P. eryngii* [12]. In our study, pathogenicity was not consistent within species (e.g., *T. pleuroticola* KACC44535 and KACC44536 showed relatively moderate and severe pathogenicity, respectively). These results reinforce the notion that *T. harzianum* and *T. aggressivum* are closely related but exhibit a broad pathogenicity range [28].

Earliness is also economically important because this measure is related to fast turnover in a cultivation room. Although no significant difference in earliness was found between the control and six treated plots (50%), all *Trichoderma* spp. were found to delay the harvest, which might have been caused by suppression of primordia formation, and the observation of soaked mycelia indicate that the mechanism underlying this suppression might involve lysis of the mycelium (Figure 1).

We developed a rapid and accurate detection method for 11 *Trichoderma* spp. that involves a single primer set. The molecular markers TDP-F and TDP-R can detect the mycelium of *Trichoderma* not only independently (Figure 4) but also in a mixture of *Trichoderma* and edible mushrooms (Figure 5). Although detection methods for *T. harzianum*, *T. pleurotum*, and *T. pleuroticola* have been developed in previous studies [10,29,30], the detection ranges of these previously developed methods are limited. Because the 11 *Trichoderma* spp. tested in our study were found to exhibit pathogenicity to *P. eryngii*, the range of detection is important for the control of green mold. In *in silico* PCR, *Penicillium* and

Aspergillus, another green molds with weak pathogenicity, were not detected with the developed primer set. We could not exclude the possibility that the developed marker could be positive on other *Trichoderma* species besides the ones tested in this study. It would not a big problem, because the nature of *Trichoderma* is parasite of other fungi [13], thus they might be antagonistic effect on edible mushrooms.

In our study, the detection limits for *Trichoderma* spp. ranged from 500 ag to 5 fg (Figure 4). These sensitivities are similar to those of nested PCR assays with specific primers targeting *Trichoderma* spp. [29], but the method developed in our study has enhanced usability because it utilizes the standard PCR protocol. The developed primer set was able to detect a 1:10,000 (wt/wt) ratio of *Trichoderma* spp.:*P. eryngii* mycelium (Figure 5), which reveals the detection effectiveness of the developed molecular markers during the initial growth phase of *Trichoderma* spp. on the mushroom substrate. This specificity is very important because the ratio of *Trichoderma* mycelium:*P. eryngii* might be very low at the early stage of spawn running. This information will help farms reduce contamination in liquid spawn systems, which has recently increased in Korea [31,32]. The liquid spawn is applied with air pressure through a membrane filter, which might increase the risk of exposure to substantial contamination from airborne *Trichoderma* spores.

The control of *Trichoderma* can be challenging due to its features, habitats, optimal temperature, aggressive growth rate, and airborne conidia [13]. In addition, the substrate is very vulnerable after the scraping of old mycelia, which act as a barrier. Before new mycelia can grow and cover the surface of the substrate, the scraped substrate might be exposed to airborne pathogens such as *Trichoderma.* Thus, the detection and removal of pathogens at the early stage and reducing their population in mushroom farms will be the best strategy. Consequently, the molecular markers TDP-F and TDP-R will lead to better management of commercial mushroom production and prevent green mold caused by *Trichoderma* spp.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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